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SYNTHESIS OF 5-AMINOLEVULINIC ACID-[^{14}C] BY CELL-FREE PREPARATIONS FROM GREENING MAIZE LEAVES

EITAN HAREL, ERNA MELLER and MOSHE ROSENBERG
Botany Department, The Hebrew University of Jerusalem, Israel

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Key Word Index—*Zea mays*; Gramineae; maize; 5-aminolevulinic acid; greening leaves; chlorophyll.

Abstract—The conversion of 2-ketoglutarate-[^{14}C] to 5-aminolevulinic acid-[^{14}C] (ALA) by a cell-free system from maize leaves is described. Optimal conversion was achieved at pH 6.2 using a 20000 g supernatant after gel filtration through Sephadex G-25. The formation of ALA required Mg^{2+}, an amino donor (alanine or glutamate), pyridoxal phosphate and NADH. NADPH was somewhat less effective.

INTRODUCTION

5-Aminolevulinic acid (ALA)* is the first committed intermediate in chlorophyll biosynthesis, but its formation has not been adequately studied in cell-free systems from plants [1, 2]. It appears that ALA is synthesized in greening leaves from the intact carbon skeleton of 2-ketoglutarate (2-KG) or glutamate [3—7], contrary to the pathway established for animal tissues, yeast and bacteria [1]. Gough and Kannangara [8] reported recently that isolated spinach chloroplasts synthesized ALA-[^{14}C] from labeled 2-KG in the presence of levulinic acid. Isolated plastids from greening cucumber cotyledons converted glutamate to protoporphyrin IX [9] and labeled glutamate to ALA-[^{14}C] (Weinstein and Castelfranco, personal communication). The accumulation of ALA by cucumber cotyledons was enhanced by ATP, GSH and levulinic acid. Both the cucumber and spinach systems were markedly dependent on the integrity of the plastids. It is therefore difficult to assess whether the effect of the various 'cofactors' is due to their involvement in the enzymic reaction or to indirect effects, e.g. maintenance of plastid integrity.

Lohr and Friedmann [10] reported the partial purification of two enzymes from maize leaves, which could, when present together, catalyze the formation of ALA from 2-KG. However, they employed extremely high concentrations of substrates and cofactors. Their communication lacked information on the contribution of the various cofactors and about quantities and the extent of labeling of intermediates and products, whose identification was not unequivocal.

More information on cell-free systems is needed in order to isolate and characterize the enzyme(s) involved in ALA synthesis in higher plants. We report on the cofactor requirement of a soluble system from greening maize leaves which catalyzes the formation of ALA-[^{14}C] from labeled 2-KG.

* ALA—5-aminolevulinic acid; 2-KG—2-ketoglutaric acid.

RESULTS AND DISCUSSION

Crude homogenates of greening leaves of maize, pea, and barley converted 2-KG-[5-^{14}C] to ALA-[^{14}C] in the reaction mixture described in Experimental. The activity was stimulated by NADH and to a lesser extent by NADPH (Table 1). Most of the activity was recovered in the 300 *g* for 5 min or 20000 *g* for 20 min supernatants (Tables 1 and 2), with little or no activity in the corresponding precipitates. The work was continued only with maize leaves, because of the large amount of information available on greening and ALA accumulation in this tissue [5, 11–14].

When maize leaves were ground in buffer containing 0.5 M glycerol or sorbitol, about 20% of the activity of the crude homogenate were recovered in the 1000 *g* for 5 min precipitate, after resuspension in buffer containing glycerol. Much less activity was recovered when the precipitate was resuspended in the absence of glycerol (Table 2). Almost 40% of the activity in the 20000 *g* supernatant were lost upon centrifugation at 100000 *g* for 1 hour (Table 2). The lost activity was not recovered in the precipitate.

Gel filtration of the 20000 *g* supernatant through a column of Sephadex G-25 resulted in a much improved conversion of 2-KG to ALA (Table 2). The increase in

Table 1. Synthesis of ALA-[^{14}C] from 2-ketoglutarate-[5-^{14}C] by cell-free preparations from greening leaves of higher plants.

Plant	Fraction	10^3 cpm in ALA pyrrole/g fr. wt/hr		
	Addition:	none	NADH (2 mM)	NADPH (2 mM)
Pea	300 *g* supernatant	10.6	20.4	17.1
	20000 *g* supernatant		23.1	23.2
Barley	300 *g* supernatant	14.0	24.2	20.2
	20000 *g* supernatant		28.0	23.6
Maize	300 *g* supernatant	16.6	30.0	17.4
	20000 *g* supernatant	13.3	26.5	16.2

Table 2. ALA synthesizing activity in various fractions from greening maize leaves

Fraction	10^3 cpm in ALA pyrrole/hr g fr. wt	mg protein	pmol ALA/hr/mg protein
(A) Leaves ground in buffer containing 0.5 M glycerol			
Crude homogenate	28.3	1.11	310
1000 *g* ppt. resuspended in buffer with glycerol	5.8	1.61	451
1000 *g* ppt. resuspended in buffer without glycerol	1.9	0.59	165
1000 *g* supernatant	21.9	0.89	249
20000 *g* supernatant	20.8	0.97	272
(B) Leaves ground in buffer without glycerol			
Crude homogenate	28.9	0.96	269
20000 *g* supernatant	26.0 ± 0.5*	1.03 ± 0.03	288 ± 9
100000 *g* supernatant	16.4	0 92	257
20000 *g* supernatant after gel filtration in Sephadex G-25	45.2 ± 2 3*	2.88 ± 0.11	806 ± 31

* Mean and s.e. of 6 independent experiments.

ALA synthesizing activity observed after gel filtration was apparently due to the removal of a low molecular weight compound(s) from the enzyme preparation. Maize leaf extracts catalyzed a rapid degradation of ALA when incubated in the conditions of the assay. About 25 nmol were decomposed per min by an extract equivalent to 500 mg leaves, when 200 nmol ALA were added to the reaction mixture in the absence of NADH. Most of this ALA-degrading activity was non-enzymic and depended on the presence of pyridoxal phosphate. More than 90% of this activity were eluted from a Sephadex G-25 column along with the low molecular weight fractions, upon gel filtration of the 20000 *g* supernatant.

Fig. 1 shows some additional properties of the cell-free system which converts 2-KG to ALA. It had an optimum around pH 6.2 (Fig. 1a). The rate of ALA formation was almost linear for at least 60 min (Fig. 1b). The rate of the reaction was not linearly dependent on enzyme concentration (Fig. 1c). The shape of the curve might indicate the presence of a dissociable activator or coenzyme, or the participation of two enzymes in the reaction. Increasing the concentration of 2-KG up to 0·5 mM at a constant specific radioactivity enhanced the

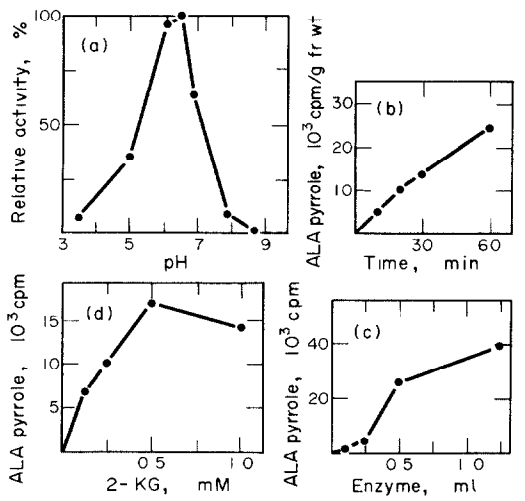

Fig. 1. Formation of ALA-[^{14}C] from 2-KG-[5-^{14}C] by a cell-free system from maize leaves. (a) The effect of the pH of the reaction mixture. (b) Formation of ALA with time of incubation. (c) The effect of enzyme concentration. (d) The effect of 2-keto-glutarate concentration at a constant specific activity (1 mCi/mmol). (a–c) using the 20000 *g* for 20 min supernatant fraction. (d) 20000 *g* supernatant after gel filtration through Sephadex G-25.

Table 3. Cofactor and substrate requirement for the synthesis of ALA by a soluble preparation from greening maize leaves

Reaction mixture	Relative activity (%) 20000 *g* supernatant after gel filtration through Sephadex G-25
Complete, 2-KG-[5-^{14}C] as substrate	100
– Enzyme	0
– Alanine	0
– Pyridoxal phosphate	30
– Mg^{2+}	10
– NADH	9
NADPH (2 mM) instead of NADH	67
Glutamate (2.5 mM) instead of alanine	107
Phenylalanine (2.5 mM) instead of alanine	2
+Glutamate (2.5 mM)	89
+ATP (2.5 mM)	83
+GTP (2.5 mM)	98
+CoA (0.5 mM) and GSH (5 mM)	96
+ALA (0.5 mM)	53
Complete, with glutamate-[5-^{14}C] (1 μCi, 0.5 mM) as substrate	68
Complete, with glutamate-[1-^{14}C] (1 μCi, 0.5 mM) as substrate	57

The 'complete' assay mixture contained (in mM final concn): K-phosphate pH 6.5 (50), EDTA (1), $MgCl_2$ (50); alanine (2.5); pyridoxal phosphate (1); NADH (2); levulinic acid (5); 1 μCi 2-KG-[5-^{14}C] (1 mCi/mmol) and enzyme (1.6–2.2 mg protein) in a final vol. of 2 ml. Activity was calculated from the radioactivity recovered in the ALA pyrrole purified from the assay mixtures, and is expressed as % of the activity in the 'complete' mixture (2625–3188 cpm in ALA pyrrole/mg protein/hr). Pooled data from six independent experiments.

rate of ALA formation (Fig. 1d). A somewhat unexpected property of the system was that the 20000 *g* supernatant from dark grown leaves was 2–3 times more active in forming ALA-[^{14}C] than preparations from leaves which had greened for several hours.

The cofactor requirement for the conversion of 2-KG to ALA is shown in Table 3. The formation of ALA required Mg ions, pyridoxal phosphate, an amino group donor (alanine or glutamate) and NADH. NADPH was less effective than NADH (Table 3). ATP, GTP, CoA and GSH had little effect on the rate of the reaction. The addition of ALA (0.5 mM) to the reaction mixture reduced the amount of ALA-[^{14}C] formed, indicating either product inhibition or reversibility of the reaction (Table 3).

The cofactors required for the conversion of 2-KG to ALA could also permit the conversion of 2-KG to glutamate by glutamate dehydrogenase or by alanine: 2-KG aminotransferase. It is therefore possible that 2-KG is converted to glutamate before being transformed into ALA. However, glutamate-[5-^{14}C] or -[1-^{14}C] were less effective precursors of ALA than 2-KG-[5-^{14}C] (Table 3). Moreover, the incorporation of label from 2-KG-[5-^{14}C] into ALA was not markedly reduced by the addition of 5 μmol glutamate to the reaction mixture (Table 3) nor was it enhanced by the addition of ammonium sulphate (40 μmol). It appears that 2-KG rather than glutamate is the immediate precursor of ALA in our system.

The conversion of 2-KG or glutamate to ALA has now been demonstrated in a number of cell-free systems from higher plants. It appears that the activity is located, at least in part, in the chloroplast ([8], Weinstein and Castelfranco, personal communication) and is partly lost upon disruption of the plastids. Our work demonstrates that the activity can be followed in 'soluble', organelle-free preparations although the possibility that it is bound to a light membrane fraction can not be excluded. The cofactor requirement for the conversion of 2-KG to ALA conforms with the report of Lohr and Friedmann [10]. These authors suggested that the formation of ALA involves two enzymes—an NADH dependent dehydrogenase, catalyzing the conversion of 2-KG to 4,5-dioxovaleric acid and an aminotransferase which transformes the latter to ALA. Such an aminotransferase was described by Gassman *et al.* in *Chlorella*, its preferred amino donors being alanine, glutamate and phenylalanine [15]. If a similar aminotransferase is operating in maize leaves, it can not use phenylalanine as a substrate (Table 3). Our results indicate that NADPH can replace NADH in the formation of ALA from 2-KG, though at a somewhat lower efficiency. Gough and von Wettstein (personal communication) have observed a preference for NADPH in a similar cell-free system from barley leaves.

The amounts of ALA formed in the *in vitro* systems from leaves described so far barely approach the limit of detection by spectrophotometric methods. They are far below the biosynthetic capabilities of intact leaves [12, 16]. ALA formation *in vitro* is probably limited by its rapid degradation and metabolism [17, 18]. Disruption of the *in vivo* organization, which might include a complex of two enzymes involved in ALA synthesis, could further lower the amounts of ALA being recovered *in vitro*. Overcoming these difficulties would greatly simplify the isolation and characterization of the enzyme(s) concerned in the biosynthesis of ALA in leaves. It should also eliminate doubts as to the contribution of contaminating microorganisms in the *in vitro* work.

EXPERIMENTAL

Etiolated maize (10-day-old), pea or barley (7-day-old) seedlings were illuminated for 3 hr with 860 lux of white fluorescent light at 22°. Leaves were harvested and ground in 50 mM K-phosphate buffer pH 6.5 (2 ml/g fr. wt leaves) containing 20 mM EDTA and 5 mM α,α′-dipyridyl, with or without 0.5 M glycerol. The homogenate was centrifuged at 300 *g* for 5 min, 1000 *g* for 5 min, 20000 *g* for 20 min or 100000 *g* for 1 hr. The ppts, resuspended in buffer, and the supernatants were assayed for enzyme activity. Gel filtration was performed on the 20000 *g* supernatant (3–4 ml) through a column (2.5 × 25 cm) of Sephadex G-25 (fine), eluting with 50 mM phosphate buffer pH 6.5. The light-green fraction which was washed-off the column at its void vol. was assayed for enzyme activity. The reaction mixture contained (mM final concn): K-phosphate pH 6.5 (50); EDTA (1); $MgCl_2$ (50); alanine or other amino acids (2.5); pyridoxal phosphate (1); NADH or NADPH (2); levulinic acid (5); and 1 μCi of either 2-KG-[5-^{14}C], glutamate-[5-^{14}C] or glutamate-[1-^{14}C] (0.5), and 1 ml enzyme in a final vol. of 2 ml. Two μmol 'carrier' ALA were added to each mixture at the end of a 30 min incubation period at 25° and the reaction was stopped by the addition of TCA to 5%. The ppt. was removed by centrifugation and the ALA-[^{14}C] purified by ion-exchange chromatography on Dowex 50W columns as previously described [5]. The ALA containing fractions were brought to pH 4.6 with M NaOAc buffer and the ALA was condensed with acetylacetone to form a pyrole [5, 11]. The pyrrole was extracted and purified as previously described and its identity and purity determined by paper- and TLC [5, 11]. Enzyme activity is expressed as cpm in the ALA pyrrole. Results were corrected for yield in preparing the pyrrole (91%) and for purity of the pyrrole as determined by paper radiochromatography. pmol ALA formed in the enzyme assay were calculated from the sp. radioact. of the labeled precursor used as substrate, assuming no contribution from endogenous substrate. The degradation of ALA by leaf extracts was followed after adding 200 nmol ALA to the reaction mixture of the assay described above. The reaction in aliquots was stopped by addition of TCA (5% final concn) and the amount of ALA was determined spectrophotometrically, after condensation with acetylacetone [11]. Radioactivity was determined by liquid scintillation counting or by radiochromatogram scanning. Protein was determined as in ref. [19]. 2-KG-[5-^{14}C] (Amersham) was dissolved in solutions of non-radioactive 2-KG to 1 mCi/mmol and used immediately or lyophilised in batches and kept under N_2 in sealed ampules at −15°. Glutamate-[5-^{14}C] and glutamate-[1-^{14}C] were purchased from New England Nuclear.

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